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Flow Cytometry based 'Acute Myeloid Leukemia Maturity Score' is a Novel Marker for Predicting Relapse in Acute Myeloid Leukemia: A Prospective Observational Study

Dr Priyanka Mishra[1], Dr Seema Tyagi[2], Dr Preeti Tripathi[3], Dr Rahul Sharma[4], Dr H P Pati[5], Dr Manoranjan Mahapatra[6]

[1]DM Hematopathology, Command Hospital, Lucknow, Uttar Pradesh

[2]MD Pathology, Trained in Hematopathology, Professor & Head, Department of Hematology, New Delhi

[3]DM Hematopathology, Department of Pathology, Air Force Hospital, Bangalore

[4]PhD, Assistant Professor, Department of Hematology, All India Institute of Medical Sciences, New Delhi

[5]MD Pathology, Trained in Hematopathology, Professor, Department of Hematology, All India Institute of Medical Sciences, New Delhi

[6]MD Medicine, Trained in Clinical Hematology & BMT, Professor, Department of Hematology, All India Institute of Medical Sciences, New Delhi

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Corresponding author: Dr Priyanka Mishra
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Abstract:

Objectives: A novel flow cytometric 'AML Maturity Score (AMS)' classifying patients into AML-immature (AML-im) and AML-mature (AML-ma) based on CD34, CD117 and Tdt expression on blasts correlated with induction remission, relapse free survival and overall survival in previous studies. We aimed to study the correlation between AMS and ELN risk group and outcomes- induction remission (IR), time to remission (TTR) and relapse.

Material and Methods: This was a combined prospective and retrospective study of 104 AML patients over 30 months. Flow cytometry based quantitative expression of CD34, TdT and CD117 on blasts was used for calculating AMS and additional tube for $CD34^{+}CD38^{-}CD123^{+}$ cells was put at diagnosis in 47 patients prospectively studied. The patients received 1-2 cycles of standard induction (3+7) comprising Daunorubicin (60mg/m^2/day for 3 days) and Cytarabine (continuous infusion 100mg/m^2/day for 7 days). Data was collected on the clinical and laboratory parameters, cytogenetics and molecular profile of these patients who were followed up for IR, TTR and relapse.

Results: Of 104 patients, AML-im were 86.6% (90/104) and AML-ma were 13.4% (14/104). Patients were classified as – favorable risk (28.8%), intermediate risk (50%) and adverse risk (21.1%) on the basis of ELN 2017. Of 104 patients, 74 attained IR and of 65 patients followed up over mean period of 18 months, 27 relapsed. The mean AMS was significantly more in patients who attained IR (p=0.003). On classifying patients as AML-im and AML-ma, TTR (p=0.000) and relapses (p=0.043) were significantly higher in AML-im group.

Conclusion: AMS is a novel flow cytometry based potential marker for predicting relapses in AML patients.

Keywords: AML maturity score, relapse, induction remission, leukemia stem cells

Introduction:

Acute Myeloid Leukemia (AML) is a heterogeneous disease with a variable prognosis. In addition to the existing European Leukemia Net (ELN) and National Comprehensive Cancer Network (NCCN) risk stratification systems based on cytogenetics and molecular profile, various studies have focused on expression of single antigens on blasts but lacked any significant correlation with prognosis [1-3]. A novel flow cytometric 'AML Maturity Score (AMS)' was designed by Schneider et al classifying patients into AML-immature (AML-im) and AML-mature (AML-ma) groups based on expression of CD34, CD117 and Tdt on the blast cells [4]. It was found to correlate with prognosis in newly diagnosed AML, particularly, the intermediate-risk group of ELN stratification and thus, directing a risk-adapted post-remission therapy [5]. They also hypothesized that the prognostic significance of the 'AMS' may be due to an increased frequency of leukemic stem cells (LSCs) within AML-im group which are chemotherapy resistant and subsequently cause relapse [6,7].

The aim of our study was to study correlation between AML maturity score and clinical outcomes in terms of induction remission (IR), time to remission (TTR) and relapse. We also studied the differences in the proportion of $CD34^{+}CD38^{-}CD123^{+}$ LSCs and clinical outcomes between the AML-im and the AML-ma group.

Material and Methods

This was a combined prospective and retrospective study carried out at the department of Haematology of a tertiary care hospital after obtaining consent from Institute Ethics Committee (letter no IECPG 98/2017 dt 22 Mar 2017). All pediatric and adult patients diagnosed as AML during May 2017 to November 2019 were consecutively recruited in the study. Among these patients, those who died prior to receiving induction or could not complete induction were excluded from the study. In addition, case files of 67 consecutive patients were retrieved from May 2015 to April 2017, of which 10 patients who could not receive complete induction were excluded from the study. Patients of acute promyelocytic leukemia , Mixed phenotype acute leukemia (MPAL) and patients presenting with relapse were excluded from the study. 3mL of the aspirate was collected in EDTA vacutainer for immunophenotyping and molecular studies and 3 mL in heparin vacutainer for karyotyping. PCR was done for *NPM1*, *CEBPA* and *FLT3-ITD* (including allelic ratio) and *BCR-ABL1* mutation. In addition to these cases, AML patients who underwent treatment at our centre in last two years were also included in the study and relevant clinical and laboratory data was retrieved from case files.

Immunophenotyping of Acute Leukemia Patients:

Flow Cytometry with Acute Leukemia panel was done by multi-parametric 6-colour flow cytometry using the markers- CD45, CD13, CD33, CD10, CD19, CD117, CD34, HLA-DR, CD7, cMPO, cCD79a, cCD3, Tdt, CD64, CD11c/CD5. The technique of 'stain-lyse-wash' was done for membranous antigens and 'lyse-perm-stain-wash' for cytoplasmic markers. An additional tube comprising CD45, CD34, CD38 and CD123 for leukemia stem cells was analyzed in patients diagnosed with AML.

Preparation of the stained tube and unstained tube for LSCs:

50µL of bone marrow aspirate was taken in 5mL polystyrene round bottom tube (12x75mm) and 50 µL normal saline was added to make the volume to 100 µL. 3.5 µL each of CD45-PerCPCy5.5 (clone H-130, Bio Legend), CD34-PECy7 (clone 581, BioLegend), CD38-APC (clone 952) and CD123-FITC (clone 6H6-BioLegend) were added to the tube and incubated in the dark for 30minutes. After incubation, 2 mL

lyse fluid was added and sample vortexed and kept in the dark for 10 minutes. After this, the sample was centrifuged for 5 minutes at 1500 rpm and the supernatant discarded. After this, 2mL sheath fluid was added to the residue in the tube and the sample centrifuged again at 1500 rpm for 5 minutes without any incubation. The supernatant was again discarded and the residue resuspended in 0.5mL sheath fluid and acquired.

The unstained tube was similarly prepared except that only 3.5 µL CD45 was added in the unstained tube instead of other antibodies in the stained tube.

Sample Acquisition and Analysis for LSCs;

One lakh events were acquired in the stained tube and 30,000 in the unstained tube. In the unstained tube, the events which were CD45 dim in the blast window on the CD45-SSC plot were gated and the quadrants set according to their auto-fluorescence.

In the stained tube, first the doublets were excluded on the FSC-H vs FSC-A plot and then the debris was excluded on the CD45 vs SSC plot. The CD34 positive events were then gated on the SSC vs CD34 plot then they were backgated on the CD45 vs SSC plot to ensure that they come from the progenitor/ blast region. The CD34positive and CD38 negative events were then selected on the CD34 versus CD38 plot and then sequential gating was done on the SSC versus CD123 plot to assess the percentage of events positive for CD123 in the CD34 positive CD38 negative population which corresponded to our LSCs. The LSCs were expressed as a percentage of blasts.

The quadrants for the LSCs were set according to the unstained tube and bone marrow aspirates of 7 Immune Thrombocytopenia patients to delineate the area in which the normal progenitors lie.

Calculation of AMS and Treatment:

'AMS' as proposed by Schneider et al [4] was calculated using the 3 markers- CD34, CD117 and Tdt as shown in Table 1. According to the total quantitative antigen expression of these markers, AML was classified as AML-mature (ma) if the score was 0-1 and AML-immature (im) if the score was 1.5-5.

Table 1: Antigen Expression for AML Maturity Score (AMS) [4]

Antigen	Expression (%)	Maturity Sore
CD34	0-19 20-49 ≥50	0 1 2
CD117	0-19 20-49 ≥50	0 0.5 1
Tdt	0-19 20-49 ≥50	0 1 2

All the patients in our study received standard induction (3+7) comprising Daunorubicin (60mg/m^2/day for 3 days) and Cytarabine (continuous infusion 100mg/m^2/day for 7 days). The patients who did not attain remission after first cycle of induction were subjected to repeat 3+7. These patients were given Tab Levofloxacin 500 mg once a day and Syrup Posoconazole 200mg three times a day as anti-microbial prophylaxis from the day of induction till breakthrough fever. Bone marrow aspirate and biopsy were done at count recovery or between day 28-35 if the absolute neutrophil count and platelets did not recover. Induction Remission was defined as attainment of Complete Remission/ Complete Remission with incomplete count recovery (CR/CRi) as per the ELN guidelines. Complete Remission

was defined as meeting all of these criteria- < 5% blasts on peripheral blood and bone marrow, no Auer rods in blasts, absence of extra-medullary disease, ANC ≥1000/µL and platelet count ≥ 100 000/µL. CRi was defined as all the above mentioned criteria for CR except ANC <1000/µL or platelet count < 100 000/µL [5]. Time to Remission was defined as the time taken to attain CR/CRi. The consolidation therapy was high dose cytarabine at dose ranging from 1.5g/kg/m2 to 3g/kg/m2 twice a day on days 1,3 and 5. The number of cycles ranged from 1-3 depending on the patient's co-morbidities and risk group. Patients not eligible for transplant were given 3 cycles of consolidation. Patients in ELN intermediate and adverse risk were offered allogeneic hematopoietic stem cell transplant after induction if they had an HLA matched sibling. Relapse was defined as recurrence of disease after CR characterised by ≥ 5% blasts on peripheral blood or bone marrow and/or extramedullary disease. Early relapse was defined as relapse within 18 months of induction remission and any relapse occurring thereafter was termed late relapse [8].

Data was collected on the clinical profile, laboratory parameters, cytogenetics and molecular profile of these patients and these patients were followed up for IR, TTR and relapse. We studied for any correlation between AMS, AML-im and AML-ma and the proportion of LSCs, ELN 2017 risk stratification group and clinical outcomes.

Statistical Analysis :

Statistical analysis was done using SPSS software version 21. Quantitative variables were compared using Independent t test/Mann-Whitney Test between the two groups and ANOVA/Kruskal Wallis test between more than two groups. Qualitative variables were correlated using Fisher's Exact test. Spearman rank correlation coefficient was used to assess the association of various parameters with AMS. Kaplan Meier survival analysis curve was used to find out OS and log rank test was used to compare overall survival between AML-im and AML-ma.A p value of <0.05 was considered statistically significant.

Results

Our study comprised of total of 104 patients of which 47 were studied prospectively and 57 retrospectively. A total of 30 patients died during induction of which 12 were part of prospective study and 18 were retrospective. Of the 39 retrospective patients who survived induction and were followed up, 3 died during consolidation. The age of the study population ranged from 4-66 years with a median age of 27 years. Majority of the patients were males with a male: female ratio of 1.4:1. The patient characteristics are as shown in Table 2. On the basis of AMS, 86.6% (90/104) patients were AML-im and 13.4% (14/104) were AML-ma. On follow-up, 74 patients attained induction remission of which 27 relapsed. Four patients proceeded to allogeneic hematopoietic stem cell transplant after induction.

Table 2: Patient Characteristics of 104 AML patients

S No	Characteristics	Patients (n=104)
1	Age	Median- 27 years (range 4-66 years)
2	Sex	Males- 60, Females- 40, M: F= 1.4:1
3	TTR [A]	Median- 2 months, range 7 days-6 months
4	ELN[B] 2017 risk group	Favorable- 30 (28.8%) Intermediate- 52 (50%) Adverse- 22 (21.1%)

5.	AML[C]-immature AML-mature	90 (86.5%) 14 (13.4%)
6.	Induction remission No Induction remission	74 30
7.	Relapse Early (< 18 months from diagnosis) Late (> 18 months from diagnosis)	27 24 3

Footnotes: A- Time To Remission, B- European Leukemia Network, C- Acute Myeloid Leukemi

The baseline cytogenetics and molecular profile of our patients is as shown in Supplementary Table 1. The majority of patients had normal cytogenetics and molecular profile (52/104) followed by *NPM1* mutated patients with *FLT3-ITD wild type* (14/104) and patients with wild type *NPM1* and mutated *FLT3-ITD* with high allelic ratio (11/104). On the basis of ELN 2017 risk stratification system for AML, patients were classified as – favorable risk (28.8%), intermediate risk (50%) and adverse risk (21.1%). We studied the correlation between AMS and clinical outcomes in terms of induction remission, time to remission and relapse as shown in Table 3. There was statistically significant correlation between AMS and attainment of induction remission (IR) (p=0.003) and time to remission (TTR) (p=0.000). The Spearman's correlation coefficient between AMS and TTR was significant (r_s=0.389) .

Table 3: Correlation between AMS and clinical outcomes

		AMS	p-value
Induction Remission (IR)	Non-IR	Median= 3 Mean= 3.18 ± 1.03	0.003
	IR	Median= 2.5 Mean= 2.53 ± 1	
Relapse	Relapse	Median= 3 Mean= 2.8 ± 0.79	0.135
	No relapse	Median= 2 Mean= 2.4 ± 1.19	
Time to Remission (TTR)	19-64days	Spearman correlation coefficient rs= 0.389	0.000

Of the total 74 patients who attained IR, 9 patients died during consolidation. The remaining 65 patients were followed up for relapse, of which 27 patients relapsed (1 AML-ma and 26 AML-im). Out of the 38 patients who did not relapse, 9 were AML-ma and 29 were AML-im. On analyzing the patients in the AML-im and AML-ma group, relapses and time to attain remission were significantly more in the AML-im group(p=0.043). The percentage of LSCs as percent of blasts in the AML-im group ranged from 0.8-97.9% with a median LSC of 29.5% (mean= 43.6%, SD=35.8). The percentage of LSCs as percent of blasts in the AML-ma group ranged from 15.8-27.8% with a median LSC of 19.1% (mean= 20.62%, SD=4.38). However, no statistically significant difference (p= 0.563) was found between percentage of LSCs as percentage of blasts between the two groups. The differences in the characteristics, LSCs proportions and clinical outcomes of the two groups is summarized as shown in Table 4.

Table 4: Difference in clinical outcomes and baseline LSCs in AML-im and AML-ma groups

S No	Parameter	AML-im (n=90)	AML-ma (n=14)	p value
1	Induction Remission (IR) Non-IR	70% (63/90) 30% (27/90)	78.6% (11/14) 21.4% (3/14)	0.345
2	Time to Remission (TTR) (days)	31	24	0.000
3	Relapse In Remission	28.9% (26/90) 32.2% (29/90)	7.1% (1/14) 64.3% (9/14)	0.043
4	Overall survival (OS)	49.44%	76.15%	0.18
5	Leukemia Stem Cells (LSCs) (%)	43.06$\pm$ 35.08 (0.8-97.9)	20.62$\pm$4.38 (15.8-27.8)	0.563

There was no significant difference in the number of patients belonging to favorable, intermediate or adverse risk group in the AML-im and AML-ma (p=0.34). In our study, out of 10 *NPM1 mut* cases, only 1 was AML-ma and 9/10 were AML-im. There were 3 cases with complex cytogenetic abnormalities, of which 2 were AML-im and 1 was AML-ma. However, all del 5q and monosomy 7 abnormalities were found in AML-im group (p=0.000). Thus, apart from del 5q and monosomy 7, there was no statistically significant difference in the distribution of the above cytogenetic abnormalities between AML-im and AML-ma (p=0.288). However, all cases of *FLT3-ITD mut NPM1 wild type* were in AML-im group (p=0.000). Both cases of biallelic *CEBPA mut* which are favorable molecular signature were present in AML-im group. Only 2/10 cases of AML with t (8:21) were found in the AML-ma group and both t (8:21) with *c-kit* positive cases belonged to AML-im group. Among AML-ma patients, 42.8% had normal cytogenetics and molecular profile whereas, these patients constituted 48.5% patients in the AML-im group. To summarize, we did not find any preponderance of low risk cytogenetic and molecular abnormalities and normal cytogenetics in the AML-ma group. High risk abnormalities such as *FLT3-ITD* mut, del 5q and monosomy 7 were exclusively found in the AML-im group.

Overall Survival in AML-im and AML-ma groups :

Among the 104 patients with a median follow-up of 26 months, the Overall Survival (OS) at 2 years was 60.4%. In the 14 patients of AML-ma group, 2 patients died at 2 years, the OS of this group being 85.7%. In the 90 AML-im patients, 38 patients died at 2 years with OS at the end of 2 years being 64.4%. Kaplan Meier survival analysis curve was used to find out overall survival and log rank test was used to compare overall survival between the 2 groups. There was no statistically significant (p=0.18) difference in the overall survival between AML-im group and AML-ma group. Of 74 patients who attained induction remission, 63 were AML-im and 11 were AML-ma. In our study, 27 of these patients relapsed on follow-up of which 1 was AML-ma and 26 were AML –im. The Relapse Free Survival (RFS) in AML-ma group was 90.9% and 41.3% in AML-im group at the end of 2 years. Kaplan-Meier analysis and log rank test for difference in relapse free survival between AML-im and AML-ma group at 2 years did not reveal any statistically significant difference between the two groups (p=0.609).

Discussion

The data on the role of flow cytometry in the prognosis of AML patients is

controversial with most of the studies focusing on single markers [1-3]. The present risk stratification systems are based on cytogenetic and molecular profile of the patients [5]. In large number of cases, the cytogenetic and molecular studies are normal and hence the patients are categorized into the prognostically variable intermediate risk group. The study by Schneider et al on AMS is the first study which studied the maturity of blasts according to expression of CD34, CD117 and Tdt and its effect on the outcome of these patients. They categorized the patients into AML-im and AML-ma on the basis of quantitative expression of immaturity markers- CD34, CD117 and Tdt and observed significantly longer relapse free survival and overall survival in the AML-ma group. They also postulated that the worse prognosis of the AML-im group could be due to higher proportion of leukemia stem cells in this group at baseline [4].

Our study is a combined prospective and retrospective observational study with a median age of 27 years and male preponderance. The patients were further risk stratified into favorable, intermediate and adverse risk groups on the basis of ELN 2017 risk stratification system. On risk stratification, majority of our patients were intermediate risk (50%) which is similar to the study by Schneider et al [4].

Of 104 patients included in the study, only 13.5% (14/104) were AML-ma and majority were AML-im (86.5%). Similarly, most of the patients in the study by Schneider et al were AML-im (60.5%).[4] In our study, the median AML maturity score (AMS) was two in the group which attained induction remission which was less than a median AMS score of three in the group which did not attain remission and this difference was statistically significant. However, Schneider et al did not study median AMS separately in these 2 groups. Instead, they studied if the number of patients who attained CR/Cri were statistically significant in the AML-ma and AML-im groups. They did not find any statistically significant difference (p= 0.115) in the CR/CRi rates between AML-ma (73.4%) and AML-im (64.1%) groups [4]. Neither did we find any statistically significant difference (p=0.345) in the CR/CRi (induction remission) rates in our AML-ma (84.6 %) and AML-im (69.2%) groups. However, we observed statistically significant difference (p=0.029) in the time to remission in the AML-im (31 days) and the AML mature (24days) groups. We also observed statistically significant correlation (p=0.000, Spearman correlation coefficient= 0.389) between TTR and AMS i.e. the time to remission was longer in patients with higher AMS.

There was statistically significant difference (p=0.043) in the number of patients who relapsed in the AML-im and AML-ma group. In our study, the difference in the AMS between the patients who relapsed and the patients who did not relapse is however not statistically significant (p=0.135). We did not find any statistically significant difference (p=0.644) in the AMS in the patients who relapsed early and those who experienced late relapse. Similarly, no statistically significant difference (p=1.000) in early and late relapse was found in the AML-im and AML-ma groups.

We did not find any statistically significant correlation in the OS between the AML-im and AML-ma groups (p=0.18). The reason why we did not experience statistically significant differences in the OS could be due to limited AML-ma (13.5%) cases in our study compared to 39.5% AML-ma cases in the study by Schneider et al [4].

Schneider et al observed that low-risk abnormalities like *NPM1 mut* AML, t (15:17) and normal cytogenetics were significantly more in the AML-ma group (p<0.001) than the AML-im group. Also, high risk cytogenetic abnormalities such as del 5q, monosomy 7, complex cytogenetic abnormalities and monosomal karyotype

were significantly more in the AML-im group than the AML-ma group ($p<0.001$) [4]. In our study, del 5q, monosomy 7 and all cases of *FLT3-ITD mut NPM1 wild type* were found in AML-im group (p=0.000). We did not find any preponderance of low risk cytogenetic and molecular abnormalities and normal cytogenetics in the AML-ma group. However, one caveat is a fewer number of cases of the AML-ma group (14/104) in our study. However, no Indian data is available with regard to the proportion of AML-ma cases in our set up.

Schneider et al summerized that the adverse prognosis in the AML-im group could be due to a possibility of higher number of LSCs in the AML-im group than the AML-ma patients [4]. We analysed the proportion of LSCs at baseline in 47 AML cases prospectively studied using CD34, CD38 and CD123 which is a marker of LSC [7]. We did not find any statistically significant difference in the percentage of LSCs as percentage of blasts (p= 0.563) between the AML-im and AML-ma groups. Also, there was no statistically significant correlation between AMS and percentage of LSCs at baseline.

The AMS is a novel concept and the data in this regard is extremely scarce. The limitations of our study are that many of the cases were retrospectively studied as the study duration was short and hence, we could not quantify LSCs in these patients. Also, the median follow-up was only 26 months which is short for studying the difference in overall survival and relapse free survival between the two groups of AML patients. A confounding factor in our study is the lack of randomization and limited molecular work-up based on ELN classification and hence data on other molecular markers which might affect the prognosis such as *DNMT3A* and *IDH* is lacking.

Conclusions

AMS is a novel flow cytometry based potential marker for predicting relapses in AML patients. Though AMS was significantly less in patients with induction remission in our study, there was no significant difference in remission rates in the AML-ma and AML-im group. Further studies with larger sample size and study duration are required to study the utility of this flow cytometry based AMS in prognosticating AML patients.

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